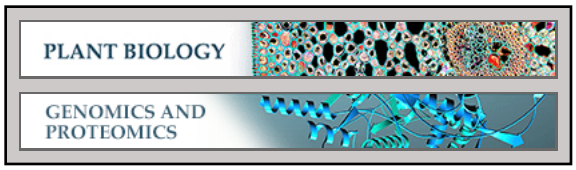
Plant Biology:

Purification and Characterization of AsES Protein: A SUBTILISIN SECRETED BY ACREMONIUM STRICTUM IS A NOVEL PLANT DEFENSE ELICITOR



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Purification and Characterization of AsES Protein

*A SUBTILISIN SECRETED BY ACREMONIUM STRICTUM IS A NOVEL PLANT DEFENSE ELICITOR**

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Background: Culture filtrates from *Acremonium* spp. induce resistance against pathogens in strawberry.
Results: A 34-kDa extracellular protein from *Acremonium strictum* exhibiting strawberry defense inducing and *in vitro* subtilisin-like proteolytic activities was characterized, and its encoding cDNA was cloned.
Conclusion: Fungal subtilisin AsES is a novel defense elicitor.
Significance: Characterization of elicitor molecules is crucial for understanding the plant-pathogen interaction and improving the plant defense response.

In this work, the purification and characterization of an extracellular elicitor protein, designated AsES, produced by an avirulent isolate of the strawberry pathogen *Acremonium strictum*, are reported. The defense eliciting activity present in culture filtrates was recovered and purified by ultrafiltration (cutoff, 30 kDa), anionic exchange (Q-Sepharose, pH 7.5), and hydrophobic interaction (phenyl-Sepharose) chromatographies. Two-dimensional SDS-PAGE of the purified active fraction revealed a single spot of 34 kDa and pI 8.8. HPLC (C2/C18) and MS/MS analysis confirmed purification to homogeneity. Foliar spray with AsES provided a total systemic protection against anthracnose disease in strawberry, accompanied by the expression of defense-related genes (*i.e. PR1* and *Chi2-1*). Accumulation of reactive oxygen species (*e.g.* H_2O_2 and O_2^-) and callose was also observed in *Arabidopsis*. By using degenerate primers designed from the partial amino acid sequences and rapid amplification reactions of cDNA ends, the complete AsES-coding cDNA of 1167 nucleotides was obtained. The deduced amino acid sequence showed significant identity with fungal serine proteinases of the subtilisin family, indicating that AsES is synthesized as a larger precursor containing a 15-residue secretory signal peptide and a 90-residue peptidase inhibitor I9 domain in addition to the 283-residue mature protein. AsES exhibited proteolytic activity *in vitro,* and its resistance eliciting activity was eliminated when inhibited with PMSF, suggesting that its proteolytic activity is required to induce the defense response. This is, to our knowledge, the first report of a fungal subtilisin that shows eliciting activity in plants. This finding could contribute to develop disease biocontrol strategies in plants by activating its innate immunity.

Anthracnose caused by fungal species of the genus *Colletotrichum* represents one of the major fungal diseases in strawberry (*Fragaria* × *ananassa* Duch.) crops (1). Anthracnose can affect all plant tissues, *e.g.* fruits, flowers, leaves, runners, roots, and crowns, and the typical symptoms are described as irregular and black leaf spot, flower blight, and fruit and crown rot, bringing about serious losses in plant and fruit productions (2).

We have previously reported a typical incompatible interaction between an avirulent isolate of *Colletotrichum fragariae* and strawberry plants of the cultivar (cv.) *Pájaro* (*Fragaria* × *ananassa*), and when strawberry plants were inoculated with this isolate prior to the challenge with a virulent strain (M11) of *Colletotrichum acutatum*, the former avirulent pathogen prevented the development of disease symptoms (3). Further studies indicated that the anthracnose resistance was accompanied by the activation of plant defense mechanisms, including the production of reactive oxygen species (ROS)[5] (*i.e.* H_2O_2 and O_2^-), accumulation of salicylic acid, and transcriptional induction of pathogen-responsive genes encoding for pathogenesis-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) JX684014.
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[5] The abbreviations used are: ROS, reactive oxygen species; CF, culture filtrate; DAMP, damage-associated molecular patterns; dpi, day post-inoculation; dpt, day post-treatment; DSR, disease severity rating; hpt, hour post-treatment; IEF, isoelectrofocusing; IR, induced resistance; PAMP, pathogen-associated molecular patterns; *p*NA, *p*-nitroanilide; PR, pathogenesis-related; PS, phenyl-Sepharose; RACE, rapid amplification of cDNA ends; RH, relative humidity; Suc-AAPF-*p*NA, succinyl-Ala-Ala-Pro-Phe-*p*NA; NBT, nitro blue tetrazolium; RP, reverse phase; PAS, periodic acid-Schiff.

related (PR) proteins (4, 5). Recently, we have also shown that strawberry plants pretreated with culture filtrates derived from that avirulent isolate also acquired the resistance to the M11 isolate, confirming that this protection effect was due to a defense response induced by one or more proteinaceous elicitors contained in these extracts (6).

Plants have an innate immunity system to defend themselves against pathogens (7). The term elicitor is commonly applied to agents stimulating any type of defense response in intact plants or cultured plant cells, resulting in enhanced resistance to the invading pathogen (8). Elicitors of a diverse chemical nature have been characterized, including (poly)peptides, glycoproteins, lipids, glycolipids, and oligosaccharides, which can be derived from a variety of different phytopathogenic microbes (9, 10) or host plants (11).

The activation of plant defense in incompatible plant-microbe interactions results from recognition by the plant of either cell surface components or molecules constitutively secreted by the pathogen or factors that are produced in the plant/pathogen interface upon contact with the host plant (10). With the primary immune system, plants can recognize pathogen-associated molecular patterns (PAMPs) of potential pathogens that activate a basal defense response (12). PAMPs, previously called "general elicitors," are highly conserved molecular structures unique to microbes that play an essential role in the microbial lifestyle (13). Several PAMP-like proteins have been identified in plant pathogens, including flagellin and "harpins" from bacteria, xylanase from fungi, invertase from yeast, Pep13 and elicitins from oomycetes, and also NEP-like proteins that are widely distributed in diverse species (10). Furthermore, many phytopathogenic fungi secrete a mixture of hydrolytic enzymes (*e.g.* cellulases, xylanases, pectate lyases, proteases, polygalacturonases, and cutinases) to degrade and traverse the outer structural barriers of plant tissues. The products generated by the hydrolysis of plant components may function as "endogenous elicitors" and are called damage-associated molecular patterns (DAMPs) (13, 14). The classic examples of DAMPs are plant cell wall fragments released by microbial xylanases, pectate lyases, and endopolygalacturonases and cutin monomers generated by cutinases (13).

Filamentous microorganisms, such as fungi and oomycetes, secrete an arsenal of effector proteins that enable parasitic infection frequently by suppressing PAMP-triggered immune responses (8, 12, 15, 16). However, these molecules can serve as elicitor signals for the plant to reinforce its defense when are specifically perceived by host cognate resistance proteins and are known as avirulence proteins or "specific elicitors" (8, 16, 17). This dual activity of elicitor effectors has been broadly reported in plant-microbial pathosystems (15). The role in virulence has been shown for a few fungal effectors, including Avr2 and Avr4 of leaf-mold fungus *Cladosporium fulvum*, which inhibit tomato cysteine proteases (Rcr3, Pip1, aleurain, and TDI65) and protect chitin in fungal cell walls against plant chitinases, respectively (17).

Recent findings illustrate a diversity of effector structures and activities, as well as validate the view that effector genes are the target of the evolutionary forces that drive the antagonistic interplay between pathogen and host (18). Biochemical, genetic, and bioinformatic strategies have been applied to the identification of elicitors from fungi. However, intrinsic function of most fungal elicitors remains elusive (17). Of the vast numbers of effector proteins that have been identified, only a few have been biochemically characterized (12). GP42 is an abundant 42-kDa cell wall glycoprotein isolated from *Phytophthora sojae* with Ca^{2+}-dependent transglutaminase activity that functions as PAMP, which triggers transcriptional activation of defense genes, accumulation of phytoalexins in parsley, and cell death in potato (19). *Phytophthora* elicitins encode structurally related small (<150 amino acids) extracellular proteins, which can induce a local necrosis called the hypersensitive response and a systemic acquired resistance in tobacco (8). It was demonstrated that class I elicitins (INF1) can bind sterols, such as ergosterol, and function as sterol-carrier proteins. In addition, phospholipase activity was assigned to elicitin-like proteins from *Phytophthora capsici*, suggesting a general lipid binding/processing role for the various members of the elicitin family (20). PcF is a secreted 52-amino acid peptide of *Phytophthora cactorum* that produces necrosis in tomato and strawberry. PcF was proposed to function as a toxin because it triggers responses that are similar to disease symptoms in host plants (21). Likewise, a dual function was found for CBEL (cellulose binding, elicitor, and lectin-like), a 34-kDa cell wall protein that was first isolated from *Phytophthora parasitica* var. *nicotianae*. It was reported that CBEL can elicit necrosis and defense gene expression in tobacco plants and, on the other side, is required for the attachment to cellulosic substrates such as plant surfaces (22). Therefore, deciphering the biochemical activity of elicitors to understand how pathogens successfully colonize and grow in their host plants has become a driving paradigm in the field of fungal pathology.

Because a similar protection effect against *C. acutatum* (M11), as described previously by Chalfoun *et al.* (6), was observed when strawberry plants were treated with culture filtrates of an avirulent isolate of the strawberry pathogen *Acremonium strictum* (SS71), we were interested to identify and biochemically characterize the defense elicitor secreted by the SS71 isolate that was also able to induce a systemic resistance against anthracnose in strawberry. The relationship between the intrinsic biochemical function of the elicitor and its ability to elicit a defense response is discussed.

EXPERIMENTAL PROCEDURES

Plant Material

Strawberry plants (*Fragaria* × *ananassa* Duch.) of the cv. *Pájaro* used in experiments were kindly provided by the strawberry BGA (Strawberry Active Germplasm Bank at National University of Tucumán). Healthy plantlets were obtained from *in vitro* cultures in MS medium (Sigma), rooted in pots with sterilized substrate (humus and perlite, 2:1), and maintained at 28 °C, 70% relative humidity (RH), with a light cycle of 16 h (white fluorescent, 350 μmol photons m^{-2} s^{-1}).

Arabidopsis thaliana ecotype Col-0 seeds were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH) and germinated on MS medium plates supplemented with 1% sucrose and 0.8% agar. Seedlings at the two-leaf stage were

then transplanted to soil and grown in a growth chamber (Sanyo) with 70% RH, under a regime of 16 h of light (white fluorescent, 200 μmol photons m^{-2} s^{-1}) at 23 °C (23). Plants of 4–5 weeks old were used for the experiments.

Fungal Cultures

Three local fungal isolates characterized in our laboratory were used in this paper as follows: M11 of *C. acutatum* (3–6); B1 of *Botrytis cinerea* (5), and SS71 of *A. strictum*. M11 and B1 isolates are preserved in the culture collection available in the Instituto Superior de Investigaciones Biológicas (Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional de Tucumán), and the SS71 isolate was deposited in the International Microorganism Bank DSMZ (Germany) under accession number DSM 24396. Fungal isolates were propagated on potato dextrose agar as indicated previously (6) and maintained on potato dextrose agar slants at 4 °C. To obtain elicitor, the SS71 isolate was cultivated in potato dextrose broth at 28 °C under continuous white fluorescent light (200 μmol photons m^{-2} s^{-1}) without agitation for 21 days.

Elicitor Protein Purification

Elicitor protein was purified from 4 liters of 21-day-old SS71 *A. strictum* culture grown in potato dextrose broth. Mycelia and spores were removed from the exhausted medium by centrifugation at 10,000 × *g* for 30 min at 4 °C, followed by filtration through diatomaceous earth (Sigma) and membranes of 0.22 μm diameter (Millipore). This axenic supernatant is referred to as the nondiluted culture filtrate (CF 1×). The extract was concentrated 10-fold under vacuum and filtrated under gas nitrogen pressure through a molecular sieve (30-kDa cutoff, Amicon). The retentate over the membrane was recovered by washing the membrane surface with 20 mM Tris-HCl buffer, pH 7.5, and was subjected to two steps of chromatographic separation by anionic exchange and hydrophobic interaction.

Q-Sepharose Fast Flow Chromatography—The fraction adjusted to pH 7.5 was loaded onto a Q-Sepharose fast flow column (Q-FF 1 ml, Amersham Biosciences) previously equilibrated with 20 mM Tris-HCl, pH 7.5 (buffer A). After washing the column with buffer A, the elution was carried out with a discontinuous increasing gradient of NaCl with buffer A containing 1 M NaCl (buffer B). A 1 ml/min flow rate was maintained. The gradient conditions were as follows: 0% buffer B for 5 min; 0–24% B in 3 min; 24% B for 8 min, 24–38% B in 2 min, 38% B for 10 min, 38–100% B in 6 min, and then held at 100% B for 10 min. Eluted fractions were combined in four Q-FPLC pools, dialyzed (12-kDa cutoff, Sigma) against bidistilled water during 24 h at 4 °C, concentrated under vacuum (SpeedVac, Savant), and assayed on strawberry plants as described below. The Q-FPLC pool that showed eliciting activity was used to dissolve $(NH_4)_2SO_4$ up to a final concentration of 1.5 M.

Phenyl-Sepharose High Performance Chromatography—This active Q-FPLC pool was applied to a FPLC phenyl-Sepharose high performance column (PS-HP 5 ml, GE Healthcare), previously equilibrated with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1.5 M $(NH_4)_2SO_4$ (buffer A). The column was eluted at a flow rate of 1 ml/min with a discontinuous decreasing gradient of $(NH_4)_2SO_4$ obtained with buffers A and B (50 mM Tris-HCl pH 7.5, 1 mM EDTA). The gradient conditions were as follows: 0% buffer B for 10 min; 0–22% B in 5 min; 22% B for 5 min; 22–30% B in 4 min; 30% B for 7 min; 30–40% B in 3 min; 40% B for 7 min; 40–50% B in 3 min; 50% B for 7 min; 50–70% B in 5 min; 70% B for 5 min; 70–80% B in 4 min; 80% B for 5 min; 80–90% B in 4 min; 90% B for 5 min; 90–100% B in 4 min and then held at 100% B for 10 min. Eluted fractions were combined in 10 PS-FPLC pools, desalted, concentrated as described above, and assayed on strawberry plants as described below.

Bioassays for Eliciting Activity

Elicitor activity of either crude fraction (CF) or purified fractions was routinely assayed on strawberry plants by analyzing the accumulation of ROS (*i.e.* H_2O_2 and $O_2^{\cdot-}$) in foliar tissue and by evaluating the systemic resistance against the M11 isolate (see below).

For biological experiments, protein concentration of CF and the pooled fractions from the purification steps were adjusted to 2.5 μg of protein ml^{-1} with sterile bidistilled water. In all cases, CF from M11, buffers, and bovine serum albumin (BSA; 10 μg ml^{-1}) were used as negative controls, and the axenic CF from SS71 as positive control.

Oxidative Burst—Accumulation of hydrogen peroxide was detected by a peroxidase-dependent *in situ* histochemical staining procedure using 3,3′-diaminobenzidine according to Thordal-Christensen *et al.* (24) and superoxide ion using a superoxide-dependent reduction of nitro blue tetrazolium (NBT) according to Doke (25). Analyses were performed on plant leaves sprayed with each of the purified fractions (2.5 μg of protein ml^{-1}) of CF SS71, 4 h after leaf aspersion, as described previously (6).

Induced Resistance (IR) against Anthracnose Disease—To evaluate the resistance inducing activity of the different fractions, plants of the cv. *Pájaro* received a double treatment (6). Briefly, only the youngest completely expanded leaf of each plant (isolated from the rest of the plant) was sprayed with each of the fractions purified from CF (or CFs from SS71 or M11 as controls), and then after 7 days, the whole plant was inoculated with a conidial suspension (1.5×10^6 conidia/ml) of the virulent isolate M11 of *C. acutatum*. Immediately after the second treatment, plants were placed in the infection chamber at 100% RH, 28 °C, in the dark for 48 h and then returned to the growth chamber at 70% RH, 28 °C, under white fluorescent light (350 μmol photons m^{-2} s^{-1}) with a light cycle of 16 h per day, where they remained for 50 days.

Susceptibility to anthracnose disease was measured by assessing Disease Severity Ratings (DSR), according to a scale described on petioles by Delp and Milholland (26). The DSR was evaluated at 21 days post-inoculation (dpi). Experimental design and statistic analysis were achieved as described previously (6). Experimental data were analyzed with the program Statistix (Analytical Software, 1996) for Windows. The LSD test was used to determine the arithmetic mean of the DSR values (significance level, 0.05) of each IR experiment, and the analysis of variance test was used to evaluate the data dispersion with respect to the mean values.

Analysis of Proteins (SDS-PAGE and 2D-DE)

In each step of purification, the total protein content was quantified according to Bradford (27) using BSA as the standard. Protein profiles were routinely analyzed by SDS-PAGE according to Laemmli (28). Previously, protein samples were desalted through Sephadex G-25 superfine semi-dry matrix (Sigma). Aliquots containing 10 μg of desalted protein were heated to 100 °C for 5 min in a denaturating mixture (28) and loaded onto a 12% (w/v) polyacrylamide gel. Gels were run at 20 mA per gel constant current for 90 min.

To study the purity of the sample, the biologically active fraction obtained by PS-FPLC was submitted to two-dimensional PAGE. Briefly, 30 μg of desalted protein were vacuum-dried and solubilized in 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM DTT, and 0.5% biolytes (Bio-Rad). Insoluble material was removed by centrifugation (15 min at 15,000 $\times$ *g*), and the supernatant was applied onto IPG strips (7 cm, 3–10 linear pH gradient, Bio-Rad), which were rehydrated for 12 h at constant 50 V with 30 μg of protein in 125 μl. Isoelectrofocusing (IEF) was carried out in a PROTEAN IEF cell (Bio-Rad) at a constant current of 50 μA per strip until 10,000 V were reached. After IEF, the IPG strips were equilibrated according to Görg *et al.* (29). Second dimension SDS-PAGE was performed on 12% polyacrylamide gels using Mini-PROTEAN III as indicated above. Gels were stained with the fluorescent dye ruthenium II tris-bathophenanthroline disulfonate (Sypro, Bio-Rad). The images were captured, digitized, and analyzed with PDQuest™ software (Bio-Rad) (30). For each spot, the pI and the molecular mass were determined (30). The two-dimensional gel analysis was repeated three times to avoid inconsistencies. For the detection of glycoside residues, 30 μg of desalted pure elicitor protein was separated by SDS-PAGE in 12% polyacrylamide gels and revealed by periodic acid-Schiff (PAS) staining according to Segrest and Jackson (31) with minor modifications. Albumin from chicken egg white (ovalbumin; Sigma) of 45 kDa was used as glycoprotein control. To detect the sensitivity of PAS staining method to glycoside residues, different amounts of ovalbumin (1, 5, and 10 μg) were loaded in the wells.

Nano-LC and Ion Trap MS/MS Analysis of Tryptic Peptides

Protein spots were excised from the gel and then digested with porcine trypsin (Promega) using in-gel digestion according to Schevchenko *et al.* (32) with minor variations. In parallel, 100 μg of pure elicitor protein (corresponding to desalted PS-FPLC pool) was proteolytically digested under highly denaturing conditions as described by Link *et al.* (33). Both tryptic digests were vacuum-dried and used for MS analysis.

MS analysis was performed by fingerprinting of the tryptic digests, using standard methods on a Biflex III spectrometer (Bruker-Franzen Analytik, Bremen, Germany) in a positive ion reflector mode. Selected tryptic peptides were on-line injected onto a C-18 reverse phase (RP) nano-column (Discovery® BIO Wide pore, Supelco) and analyzed in a continuous acetonitrile gradient consisting of 0–50% B in 45 min, 50–90% B in 1 min (A = bidistilled water; B = 95% acetonitrile, 0.5% acetic acid). A flow rate of ~300 nl/min was used to elute peptides from the RP nano-column to a PicoTip™ emitter nano-spray needle (New Objective, Woburn, MA) for real time ionization and peptide fragmentation on an Esquire HCT IT (Bruker-Daltonics) mass spectrometer. A 3-Da window (precursor $m/z \pm 1.5$), MS/MS fragmentation amplitude of 0.90 V, and a dynamic exclusion time of 0.30 min were used for peptide fragmentation. Nano-LC was automatically performed on an advanced nano-gradient generator (Ultimate nano-HPLC, LC Packings, Amsterdam, The Netherlands) coupled to an autosampler (Famos, LC Packings). The software Hystar 2.3 was used to control the whole analytical process. MS/MS spectra were batch-processed by using DataAnalysis 5.1 SR1 and BioTools 2.0 software packages and searched against the MSDB protein database using MASCOT software (Matrix Science) or sequenced *de novo* followed by MS BLAST alignment at EMBL with default settings.

RP-HPLC and MALDI-MS Analysis of Intact Protein

The absolute purity of the native elicitor protein obtained by PS-FPLC was verified using two different methods as follows: analytical RP-HPLC and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS analysis. Briefly, 100 μg of lyophilized protein (corresponding to desalted PS-FPLC pool) was resuspended in bidistilled water and chromatographed on an HPLC μRPC C2/C18 column (GE Healthcare) using a ÄKTA purifier 10 system (GE Healthcare). The column was previously equilibrated in bidistilled water with 0.1% (v/v) trifluoroacetic acid and eluted with a linear gradient of acetonitrile with 0.1% (v/v) trifluoroacetic acid at 0.7 ml/min flow rate. Peaks were detected at $\lambda = 285$ nm. In parallel, 10 μg of lyophilized intact protein was resuspended in 50% acetonitrile with 0.1% (v/v) trifluoroacetic acid and analyzed on a Biflex III MALDI-TOF mass spectrometer with delayed extraction (Brucker-Franzen Analytik, Bremen, Germany) using standard methods. The instrument performed acquisition of a full-scan mass spectrum ranging from 500 to 100,000 Da.

N-terminal Sequencing

The biologically active purified protein was run on a 12% SDS-polyacrylamide gel and electroblotted onto a 0.1-μm pore size PVDF transfer membrane (Immobilon-P^{SQ}, Millipore) at 4 °C applying constant voltage (at 45 V) for 12 h. Transfer buffer was 10 mM CAPS (3-(cyclohexylamino)propanesulfonic acid), pH 11.0, 10% methanol, and 5 mM DTT (34). Protein bands were visualized by staining with 0.1% Coomassie Brilliant Blue-R in 50% methanol, 1% acetic acid for 1 min. The membrane was unstained with 50% methanol. The band corresponding to the elicitor protein was excised and subjected to N-terminal amino acid sequencing by automated Edman degradation on an Applied Biosystems 477A gas-phase sequencer (Applied Biosystems, Foster City, CA).

Fungal RNA Preparation

Fresh SS71 *A. strictum* mycelium (1.2 g wet weight) was harvested by filtration from the liquid culture and pestle-homogenized in liquid nitrogen. Subsequently, total RNA was obtained and purified according to Iandolino *et al.* (35). RNA quantification was performed by spectrophotometry at 230, 260, and 280 nm. Reverse transcription reactions were carried out with 5 μg

of DNase-treated total RNA, using SuperScript II RT (Invitrogen) following the recommendations of the manufacturer.

Degenerate PCR

The degenerate PCR primers used were designed from the less conserved regions of amino acid sequences *de novo* of tryptic peptides and N-terminal region of the elicitor protein, according to the following criteria: (*a*) frequency of codons usage in the 27 cDNA and 19 genes for *Acremonium* spp. reported so far, by using the program General Codon Usage Analysis, and (*b*) codons encoding identical amino acids to that sequenced in five homologous proteins of different fungal species identified by using BLASTP algorithm (36). The combinations of degenerate oligonucleotide primers N1, N2, IF, IR, C1, and C2, used in three consecutive PCRs, are represented in Fig. 3. PCRs were carried out in 25-μl total volume containing 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 2 μM of each primer, 0.2 mM of each dNTP, and 0.75 units of TaqDNA polymerase (Invitrogen). 200 ng of cDNA were used as template for the initial PCR, and 1:125 dilution from the latter PCR product was used for the first nested PCR. The amplified band exhibiting the expected size was excised from the agarose gel, purified, and used as template for both second nested PCRs (Fig. 3). PCR amplifications were performed in a PTC-100 thermal cycler (MJ Research). Cycling conditions included an initial denaturizing step of 10 min at 94 °C, followed by 40 cycles of 45 s at 94 °C (melting), 30 s at 55 °C (annealing), 1.5 min at 72 °C (extension), and a final extension step of 10 min at 72 °C.

Cloning and Sequencing

After electrophoretic separation of each nested PCR product, sharp bands of the expected size were excised from the gel and purified using the GFX™ PCR DNA and gel band purification kit (GE Healthcare). The purified cDNA obtained for each band was cloned using the plasmid pCR®4.1 of the TopoTA cloning kit (Invitrogen) following the manufacturer's recommendations and transformed into *Escherichia coli* DH5-α strain. Recombinant plasmids were extracted from 10 recombinant bacterial colonies using Wizard plus SV Minipreps DNA purification system (Promega) and digested with EcoRI to verify the presence of the expected insert. Sequences of PCR products were determined using an ABI PRISM ™ 377 DNA sequencer (Applied Biosystems) (37).

RACE Experiments

To isolate the full-length elicitor-coding cDNA, RNA ligase-mediated rapid amplification reactions of cDNA ends (RACE) were performed with the GeneRacer kit (Invitrogen) as recommended by the manufacturer, using total RNA purified from 150 mg of SS71 mycelium with the RNeasy minikit (Qiagen). cDNA synthesis was performed by SuperScript III RT (Invitrogen) from 4 μg of 5′-modified mRNA in the presence of GeneRacer oligo(dT) primer, for 3′-end extension.

Based on nucleotide multiple sequence alignment with homologous proteins, less conserved regions were identified in the partial sequence of elicitor cDNA, and a new set of specific primers were designed following GeneRacer kit instructions. The primers S-5, SN-5, S-3, and SN-3 were then used for 5′ or 3′ cDNA ends cloning through nested PCRs as depicted in Fig. 3, by using High Fidelity Platinum TaqDNA polymerase (Invitrogen). The anchor primers, R-5, RN-5, R-3, and RN-3, were part of the kit (Fig. 3).

For 5′-RACE, the single strand cDNA (1:5 dilution) was used as the template for PCR in the presence of the S-5 (0.2 μM) and R-5 (0.6 μM) primers. A nested PCR was carried out by diluting an aliquot of the previous PCR product (1:10 dilution) in a second PCR mixture containing the SN-5 and RN-5 nested primers to concentration 0.2 μM. Similarly, for 3′-RACE, the single strand cDNA was utilized as the template in the subsequent PCR and one nested PCR in the presence of the S-3 and R-3 primers and SN-3 and RN-3 nested primers, respectively (Fig. 3). First amplifications were performed using "touchdown" conditions (38) as follows: 2 min at 94 °C; 5 cycles (30 s at 94 °C; 30 s at 72 °C; 4 min at 68 °C); 5 cycles (30 s at 94 °C; 30 s at 70 °C; 4 min at 68 °C); 25 cycles (30 s at 94 °C; 30 s at 66 °C; 4 min at 68 °C), and 10 min at 68 °C. Nested PCR conditions were as follows: 5 min at 95 °C; 30 cycles (30 s at 95 °C; 30 s at 69 °C; 2 min at 68 °C) and 10 min at 68 °C. The cDNA fragments of both 3′- and 5′-end extensions obtained from RACE experiments were purified from the agarose gel and ligated by a standard cloning procedure, as described above. After transformation of the *E. coli* DH5-α strain, 10 recombinant plasmid clones were purified and subjected to automated dideoxy chain termination sequencing as described above.

Sequence Edition and Analysis

Cloned sequences were trimmed of vector sequence contamination using VecScreen at NCBI (www.ncbi.nlm.nih.gov). Assemblage of cDNA sequences and translation to the predicted amino acid sequence were performed using the DNAMAN software (version 6.0). Identity of the fungal elicitor obtained was studied by comparisons of deduced amino acid and nucleotide sequences with sequences included in the GenBank™ NR database by using BLASTX and BLASTP algorithms (36).

Determinations of conserved structural motifs and domains in sequence were carried out with CDSearch (at NCBI) (39), SMART 5 (40), and Pfam (at Sanger Institute) (41). Multiple sequence alignments were performed with Clustal X (42) and edited with BOXSHADE. The SignalP server (43) was used to predict the presence and localization of signal peptide cleavage sites. Potential *N*- and *O*-glycosylation sites were identified by using NetNGlyc 1.0 and NetOGlyc 3.1, respectively. Molecular mass and pI were calculated by using PeptideMass program at the ExPASy server.

Proteolytic Activity Determinations and Protease Inhibitor Assays

Proteolytic activity was measured in fractions that exhibited eliciting activity obtained in each purification step using the chromogenic peptides *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-*p*NA; Sigma) or N^{α}-benzoyl-DL-Arg-*p*-nitroanilide hydrochloride (Sigma), as specific substrates. Substrate hydrolysis was measured by adding 10 μl of each indicated substrate (5 mM) (final concentration of 0.1 mM) to an appropriate amount of protein sample dissolved in 20 mM Tris-

HCl, pH 7.5, for a final volume of 500 μl. Reaction mixtures were incubated at 37 °C for 30 min, and the hydrolysis of the chromogenic substrates was monitored per min at 405 nm (44). All activity assays were performed in triplicate. Subtilisin Carlsberg (subtilopeptidase A) of *Bacillus licheniformis* (Fluka) was used as reference of protease activity. The proteolytic activity of pure elicitor protein was calculated as the concentration of *p*NA liberated per min, where *p*NA concentration was determined spectrophotometrically to 405 nm using a molar $\epsilon_{405\ nm}$ of 9.62 $mM^{-1}\ cm^{-1}$, at 37 °C and pH 7.5. Autoproteolysis rate of substrate Suc-AAPF-*p*NA was evaluated and subtracted to each measured value.

For protease inhibitor studies, pure elicitor protein was preincubated with different protease inhibitors for 1 min at 37 °C, at the concentrations indicated for a final volume of 500 μl. Inhibition assays were performed against the substrate Suc-AAPF-*p*NA as described above.

Protease Inhibitor Treatment of Elicitor Protein—Pure elicitor protein (50 μg) resuspended in 1 ml of 20 mM Tris-HCl, pH 7.5, was treated with PMSF (to final concentration of 1 mM) at 37 °C for 30 min and then diluted 20-fold with the same buffer (until reaching a final concentration of 2.5 $\mu g\ ml^{-1}$). PMSF-treated elicitor protein was used in IR experiments with strawberry plants as described above. PMSF adjusted to 0.05 mM with 20 mM Tris-HCl, pH 7.5, was used as negative control, and proteinase K from *Tritirachium album* (2.5 $\mu g\ ml^{-1}$; Sigma) and subtilisin Carlsberg (2.5 $\mu g\ ml^{-1}$) were used as control of protease activity.

Expression Analysis of Defense-related Genes

Total RNA from strawberry leaves was obtained, according to the protocol published by Iandolino *et al.* (35), with some modifications. One gram of fresh young leaves was sampled from elicitor-treated plants at different time points, from 0 to 72 h post-treatment (hpt). Amount and quality of obtained total RNA were monitored, and reverse transcription reactions were performed as described above. Semi-quantitative RT-PCR method was used to evaluate the expression of plant defense response-associated genes (4). Expressions of *FaPR1* (pathogenesis related-protein 1) and *FaChi2-1* (class II chitinase) genes were studied using nucleotide sequences previously reported in GenBank™ (AB462752 and AF147091, respectively). The gene *GAPDH1* (AF421492) was used as control (housekeeping) to adjust the amount of cDNA in each treatment and to attain the same exponential phase PCR signal strength. A volume of 25 μl of reaction mix containing Green GoTaq Reaction Buffer 1×, pH 8.5 (Promega), 1.5 units of GoTaq DNA polymerase (Promega), 1.5 mM $MgCl_2$, 400 μM of each dNTPs 0.4 μM of each primer, was mixed with 5 μl of cDNA (properly diluted to adjust the amount according to housekeeping gene) in each PCR. Specific primers used were as follows: GAPDH1 (forward), 5′-CTACAGCAACACAGAAAACAG-3′, and GAPDH1 (reverse), 5′-AACTAAGTGCTAATCCAGCC-3′; FaPR1 (forward), 5′-TGCTAATTCACATTATGGCG-3′, and FaPR-1 (reverse), 5′-GTTAGAGTTGTAATTATAGTAGG-3′; and FaChi2-1 (forward), 5′-TCGTCACTTGCAACTCCTAA-3′, and FaChi2-1 (reverse), 5′-GGACTTCTGATTTTCACAGTCT-3′. The PCR program used was as follows: 7 min at 94 °C (initial step); cycles (see below) of 45 s at 94 °C, 1 min at different annealing temperature (see below), 1.5 min at 72 °C; and a final extension step of 10 min at 72 °C. The number of cycles used for each gene was adjusted to obtain the specific band amplified at the exponential phase of PCR. Annealing temperatures for *GAPDH1*, *FaPR-1,* and *FaChi2-1* genes were 52, 57, and 55 °C, respectively. Amplified bands were visualized in ethidium bromide-stained (10 $\mu g\ ml^{-1}$) agarose gel (2%) and photographed under UV light (340 nm) with a digital camera. Bands observed were digitalized and quantified using Total Lab Quant software (Nonlinear Dynamics Ltd., Newcastle, UK). Relative expression of studied genes was calculated as the change of the band intensity in treated leaves with respect to control leaves. To ensure the absence of genomic DNA in each cDNA sample, GAPDH1 primer sequences were designed to enclose an intronic region.

IR Assay against Gray Mold Disease

To evaluate the protection against *B. cinerea*, plants of the cv. *Pájaro* were sprayed with the elicitor protein purified from CF (or water as negative control), and then after 7 days were inoculated with the virulent isolate B1 of *B. cinerea* as described previously (5). Immediately after inoculation, plants were placed in the infection chamber at 100% RH, 20 °C under continuous white fluorescent light (50 $\mu mol\ photons\ m^{-2}\ s^{-1}$) for 48 h, and then returned to the growth chamber under conditions indicated above, where they remained for 50 days. Susceptibility to gray mold disease was evaluated at 10 dpi by measuring necrotic lesion extension on leaves, according to a DSR scale described by Vellice *et al.* (45). Experimental design and statistical analysis were achieved as described above (6).

Tests of Eliciting Activity in Arabidopsis

Defense responses were evaluated through histochemical staining techniques in leaves of *A. thaliana* plants sprayed with the pure elicitor protein (2.5 $\mu g\ ml^{-1}$) or distilled water as control. Leaves were collected at different times post-treatment: from 0 to 12 hpt for burst oxidative and every 3 days up to 12 days post-treatment (dpt) for callose deposition. Evaluation was performed on 20 leaf tissues obtained from five plants that were equally treated.

Hydrogen peroxide was detected using the 2′,7′-dichlorofluorescein diacetate (Invitrogen) probe according to Bozsó *et al.* (46) modified in our laboratory (4), and superoxide anion by NBT staining (25), as described previously (6). To detect callose deposition, leaves were treated and stained with 0.01% aniline blue (Sigma) according to Yun *et al.* (47). Samples were examined with an Olympus System Microscope model BX51 equipped with U-LH100HG reflected fluorescence system, setting blue excitation filter (U-MWB2). Images were captured by Olympus Video/Photo Adapter (Olympus, Hamburg, Germany), and callose depositions were quantified with Image Pro Plus software (Media Cybernetics).

RESULTS

Purification of the Elicitor Protein—Previous experiments let us to conclude that 0.4 μg of protein (referred to total protein content from CF of SS71 isolate) was the minimum amount

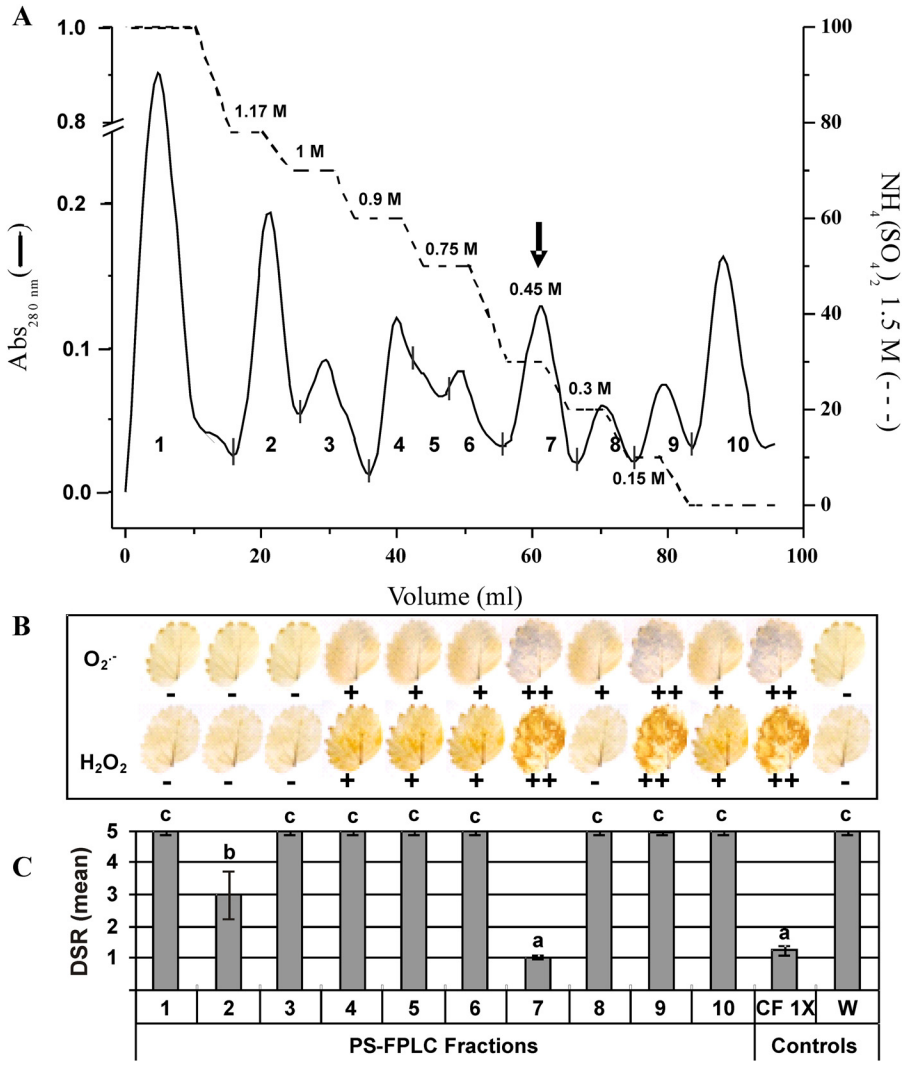

FIGURE 1. **Last purification procedure step of elicitor protein from the CF of *A. strictum* isolate SS71.** *A*, PS-high performance FPLC profile obtained from the flow-through of the previous FPLC step by Q-Sepharose column, both described under "Experimental Procedures." The *dotted line* indicates the NaCl gradient; the *solid line* indicates the absorbance at 280 nm; the *vertical marks* on the chromatograph indicate the eluted fractions combined in 10 pools; and the *arrow* indicates the pool 7 that contained the eliciting activity found in the flow-through of Q-FPLC. *B*, defense eliciting activity of PS-FPLC pools evaluated as the accumulation of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) in leaves of strawberry plants of the cv. *Pájaro*, treated with each one of the PS-FPLC pools (pools 1–10) with 2.5 μg of protein ml^{-1}, water (*W*), or CF 1× (crude fraction, protection control). Superoxide anion and hydrogen peroxide were analyzed by NBT and 3,3′-diaminobenzidine staining methods, respectively. Photos were taken at 4 h after leaf aspersion corresponding to time of the maximum of ROS production. Each micrograph represents an example of 20 leaflets analyzed coming from five plants. *C*, systemic resistance eliciting activity of PS-FPLC pools observed as the reduction of disease severity caused by the M11 isolate of *C. acutatum*. DSR were evaluated at 21 dpi on plants sprayed on one leaf with each of the fractions and 7 days later infected with the M11 isolate. *Different letters* represent statistically different DSR values (Tukey test, $p = 0.05$).

required to attain a complete suppression of anthracnose (DSR = 1) at 21 dpi. During the purification of the elicitor protein from CF, the whole eliciting activity was recovered in the flow-through fraction of the Q-FPLC step (data not shown). The profile of the PS-FPLC chromatographic separation corresponding to the last purification step is illustrated in Fig. 1*A*, and the defense eliciting activity was evaluated in all fractions by analyzing the ROS accumulation and in parallel the resistance against M11 isolate in strawberry plants of the cv. *Pájaro* (Fig. 1, *B* and *C*).

As shown in Fig. 1*B*, pools 7 and 9 of PS-FPLC induced a strong accumulation of hydrogen peroxide and superoxide anion in strawberry foliar tissue within 4 h after leaf aspersion; however, pool 7 is the unique fraction that yielded the maximum protection activity against M11 (Fig. 1*C*), similar to that observed with CF 1× from SS71 used as defense-eliciting control. Plants pretreated with pool 7 exhibited no symptom of anthracnose (DSR = 1) at 21 dpi in contrast to control untreated plants (DSR = 5) (Fig. 1*C*).

Two-dimensional PAGE analysis of the pool 7 obtained by PS-FPLC exhibited a single protein spot that migrated as a 34-kDa protein with pI of 8.8 (Fig. 2*A*), indicating that the elicitor protein was purified to homogeneity. Furthermore, the single and sharp chromatographic peak observed in the analytical RP-HPLC profile (Fig. 2*B*) and a single signal obtained from MS analysis of the pool 7 (data not shown) revealed the absence of protein contaminants that could co-purify with the elicitor in the pool 7 of PS-FPLC. However, the absolute purity was evidenced by MS/MS analysis from trypsin-digested pool 7, whereby peptides (masses and sequences) coming from a single

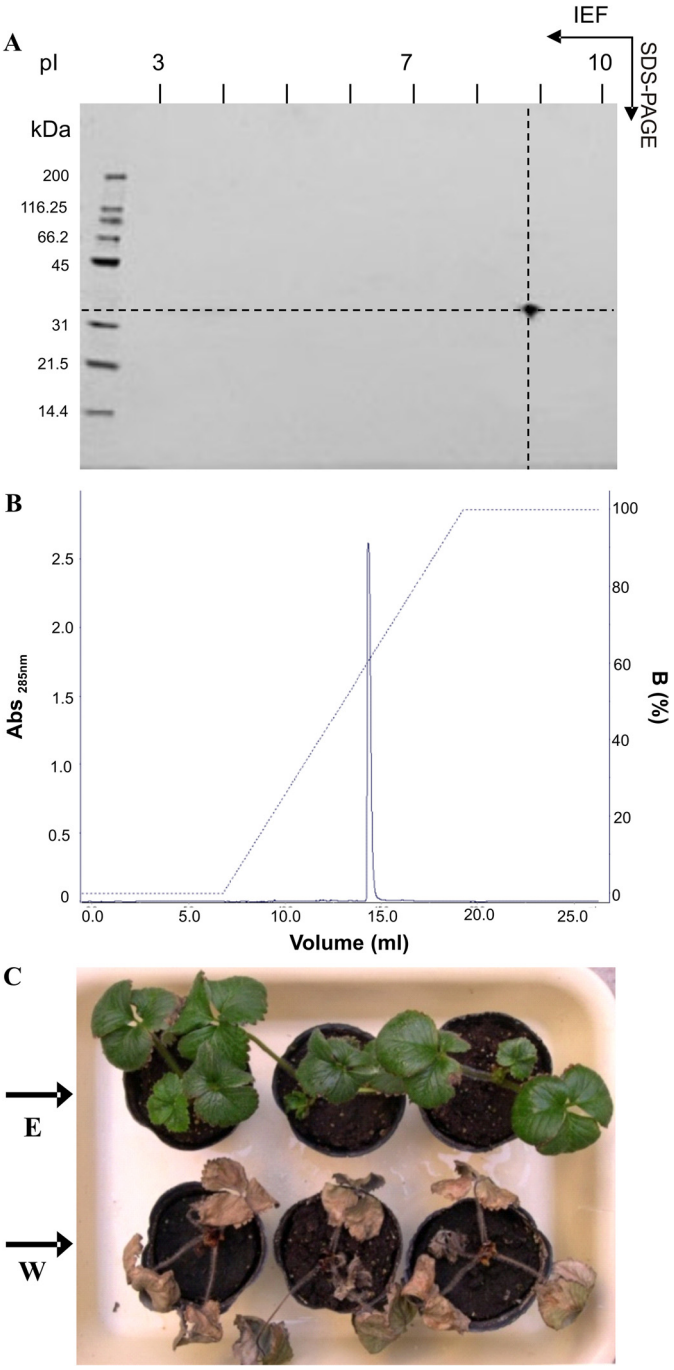

FIGURE 2. **Two-dimensional PAGE, RP-HPLC, and eliciting activity of the elicitor purified from SS71 *A. strictum* culture.** *A,* biologically active fraction obtained by PS-FPLC (30 μg of protein) was subjected to IEF in IPG strips with linear pH gradient 3–10 (first dimension), followed by SDS-Laemmli-PAGE 12% (second dimension), and finally stained with ruthenium II tris-bathophenanthroline disulfonate (Sypro). Molecular mass (in kDa) and pI standards are shown on the *left* and *top*, respectively. The *arrow* indicates the elicitor protein coordinates. *B,* RP-HPLC profile by C2/C18 column obtained from the active pool 7 PS-FPLC (100 μg of elicitor protein). The *dotted line* indicates the acetonitrile gradient (% B), and the *solid line* indicates the absorbance at 285 nm. *C,* response of strawberry plants of the cv. *Pájaro* treated with the purified elicitor (2.5 μg ml^{-1}) (E) and water (*W*) and 7 days later challenged with the M11 virulent isolate of *C. acutatum* (1.5×10^6 conidia/ml). Photograph shows three representative plants of both treatments at 21 dpi.

protein were identified after subtracting those derived from trypsin autolysis (data not shown). The resistance inducing activity of the pure elicitor protein was tested again. Fig. 2*C* shows the phenotypic aspect of strawberry plants challenged with M11 that were pretreated by spraying only one leaf with the elicitor or with water. Results show that the pure elicitor at a concentration of 2.5 μg of protein/ml was sufficient to induce a complete and systemic protection against the M11 virulent isolate. Strawberry plants pretreated with the elicitor looked healthy and vigorous at 21 dpi (Fig. 2*C*, *E*), whereas plants pretreated with water manifested a severe disease symptoms and death before 21 dpi (Fig. 2*C*, *W*).

Peptide Analyses—N-terminal sequence of elicitor protein, corresponding to the first 10 amino acids, was directly determined as AYTTQASAPW by automated Edman degradation, demonstrating that the N terminus is unblocked. Two internal tryptic peptides of elicitor, p25 and p20, were also sequenced *de novo* and yielded the following amino acid sequences: p25, VLSDSGSGSTSGIIAGINYVVSDSR, and p20, IVAIATTGVITGIPSGTPNR. The fact that the N-terminal amino acid is an alanine instead of a methionine suggests that the elicitor protein is synthesized as a larger precursor. To investigate the upstream N-terminal and the downstream C-terminal sequences, cDNA cloning and sequencing were carried out.

cDNA Cloning—The knowledge of the elicitor partial amino acid sequences allowed us to design degenerate oligonucleotide primers to identify the elicitor-coding cDNA. After each second nested PCR, single cDNA fragments of 350 or 480 bp were isolated, whose sequences corresponded to two halves of cDNA that encodes elicitor (Fig. 3, *A* and *B*).

To complete the elicitor-coding cDNA sequence, RACE experiments were undertaken, and bands corresponding to fragments of the 3′- and 5′-ends were cloned and sequenced (Fig. 3, *C* and *D*). A single 5′-RACE sequence of 680 bp and two 3′-RACE sequences of 780 and 650 bp were obtained. Both 3′-RACE clone sequences proved to be identical; however, the larger sequence showed an additional ~130-bp sequence at its 3′-end, presumably arising from a different polyadenylation site on the mRNA. All three RACE clones overlapped with the corresponding cDNA of the original fragment isolated by PCR with degenerate primers, confirming their authenticity. cDNA sequence analysis showed an ORF comprising 1167 bp that would encode for the elicitor protein of 388 amino acid residues. Fig. 4 shows the full-length cDNA sequence and the deduced amino acid sequence of elicitor protein. The N terminus and internal peptides of the elicitor previously sequenced *de novo* matched exactly the deduced amino acid sequence of the cDNA (Fig. 4). Further analysis showed that the polypeptide contained high percentages of alanine (12.1%), glycine (11.9%) and serine (11.1%), and low percentages of tryptophan (0.5%) and methionine (0.8%).

Homology Comparison—Data bank (Swiss-Prot, EMBL, and GenBankTM/EBI) surveys using the BLAST program yielded significant similarity score with precursors of extracellular serine proteases that belong to the subfamily of proteinase K-like subtilisins (S8A) and are produced by several fungal species that are members of ascomycetes and deuteromycetes. The highest values of similarity in alignments with elicitor sequence corresponded to a proteinase T-like subtilisin from the saprophyte *Trichoderma reesei* (EGR46243.1) exhibiting 65% of identical amino acids in an alignment that covered 95% of the sequence

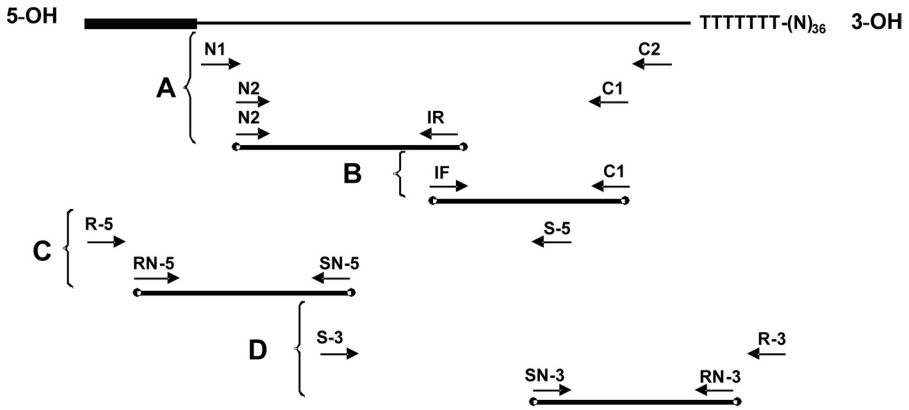

FIGURE 3. **Scheme of the isolation strategy used to obtain the elicitor-coding cDNA.** *Top*, cDNA encoding for the elicitor protein, which has been obtained by overlapping the nucleotide fragments *A–D*, identified after nested PCR with degenerate primers and RACE experiments (see details under "Experimental Procedures"). *A,* 350-bp fragment isolated from expression library by nested PCR with the combinations N1/C2, N2/C1, and N2/IR of degenerate primers. *B,* 480-bp fragment isolated from expression library by nested PCR with the combinations N1/C2, N2/C1, and IF/C1 of degenerate primers. *C,* 5′-RACE fragment of 680 bp amplified with the S-5- and SN-5-specific nested primers. *D,* 3′-RACE fragment of 780 bp amplified with the S-3- and SN-3-specific nested primers. The oligonucleotide primers utilized were as follows: *N1*, 5′-GCNTAYACNACNCARGCNT-3′; *N2*, 5′-CAGGCBWSBGCBCCBTGG-3′; *IF*, 5′-ATYATYGCYGGYATYAAC-TAYG-3′; *IR*, 5′-CRTAGTTRATRCCRGCRATRAT-3′; *C1*, 5′-RATVACRCCVGTVGTVGCRAT-3′; *C2*, 5′-GTTVGGVGTRCCVWSVGGR-3′; *S-5*, 5′-GATGTTGTTGTCGAT-CAAGGACTTGG-3′; *SN-5*, 5′-TGCCTTGGTAGGAGACAAGCTGGAA-3′, *S-3*, 5′-AGCTTGTCTCCTACCAAGGCAGCAA-3′; *SN-3*, 5′-GGCCAAGTCCTTGATCGACAA-CAAC-3′. *R-5* and *RN-5* and *R-3* and *RN-3* primers were the nested anchor primer for ends 5′ or 3′, respectively, supplied by the 3′/5′ GeneRacer kit (Invitrogen).

(*E* value, 2e-170) and also subtilisin-like serine proteases from the nematophagous fungi, *Paecilomyces lilacinus* (ABO32256.1) and *Hirsutella rhossiliensis* (ABD96101.1) presenting amino acid identities of 64 and 62% that covered 100% (*E* value, 6e-170) and 98% (*E* value, 2e-162) of the sequence, respectively.

However, the elicitor protein showed relatively low similarity (less than 45%) with the completely sequenced proteases from *Acremonium* spp., whose sequences have been inferred from homology as follows: 44.8% of identity with an alkaline proteinase precursor of 42.1 kDa (BAA00765.1) and 39.2% with a subtilisin-like serine protease of 58.37 kDa (BAF62454.1) (48). Sequence analyses revealed, however, that the elicitor protein was more similar (53.7% identity) to a cephalosporin C acetylhydrolase of *Acremonium chrysogenum* (CAB87194.1), an unspecific esterase of 38.22 kDa related to serine proteases that remove the acetyl group of cephalosporin C, although it lacks proteolytic activity (49). These data indicated that the elicitor protein is a new member of subtilisin-related alkaline protease, and it was named AsES, in reference to the producer organism *A. strictum* and their properties as elicitor and subtilisin. The AsES-coding cDNA was submitted to GenBank™ and registered under accession number JX684014.

Sequence Analysis of the Elicitor Protein—The SignalP program at the ExPASy database server predicted with high probability a cleavage site between residues 15 and 16 of the full-length pre-protein sequence, indicating the existence of a signal peptide of 15 amino acids (Met^{1}–Gly^{15}). Informatic analysis performed with CD-search (NCBI) and InterPro (EMBL-EBI) programs (Fig. 4) suggested the presence of two domains highly conserved in precursors of subtilisin-like serine proteases, a peptidase inhibitor I9 domain (Asp^{38}–Ala^{105}; E value, 2.44 e-11) that is a subtilisin propeptide, and a peptidase S8 domain (Trp^{115}–Thr^{365}; E value, 8.43 e-76) corresponding to the catalytic domain shared by all members of the subtilisin S8A subfamily that include the proteinase K-like subtilisins. AsES was composed of 388 amino acid residues, which contained a signal peptide (15 amino acids), a propeptide (90 amino acids), and a mature peptide (283 amino acids). The sequenced amino acids of N-terminal region are located at positions 106–116 of the polypeptide (Fig. 4); accordingly, we have considered the Ala^{106} residue as the beginning site of the mature protein.

In the sequence of the mature elicitor protein, the four Cys residues comprising the two disulfide bonds (Cys^{36}–Cys^{125} and Cys^{180}–Cys^{252}) found in homologous subtilisins were detected, and two putative Ca^{2+}-binding sites have been predicted (Fig. 4). The amino acids Asp^{41}/His^{71}/Ser^{226} of the catalytic triad and other amino acids of the active site (*i.e.* Leu^{135}, Gly^{136}, Asn^{163}, and Ser^{223}) are preserved in the S8 domain of AsES (Fig. 4), as in homologous subtilisins. The latter led us to speculate that AsES may also exhibit proteolytic activity.

The PeptideMass program predicted a preprotein precursor with a calculated molecular mass of 39,721 Da and a calculated pI value of 7.67 and a mature protein of 28,187 Da and pI of 7.88. These last values disagree with those obtained from the mobility in two-dimensional PAGE of purified protein (34,000 Da; pI = 8.8; Fig. 2*A*). One potential *N*-glycosylation site (Asn^{232}) and six *O*-glycosylation sites (Thr^{283}, Thr^{286}, Thr^{290}, Thr^{291}, Thr^{324}, and Ser^{387}) were predicted with low probability within the mature elicitor protein, although PAS staining on SDS-PAGE of purified AsES showed that this protein lacks glycoside residues (data not shown) in accordance with the subtilisin-like proteins reported.

Proteolytic Activity and Inhibitors—Results obtained show that the purified AsES can cleave the chromogenic tetrapeptide Suc-AAPF-*p*Na, which can be specifically hydrolyzed by subtilisins and some chymotrypsins (Fig. 5). Analysis of the proteolytic activity shows that the elicitor protein was 300-fold less effective than the control Carlsberg subtilisin in cleaving Suc-AAPF-*p*N; the specific activity of AsES at 24 h was 108 μmol min^{-1} mg $protein^{-1}$ as compared with the Carlsberg subtilisin that reached 32,812 μmol min^{-1} mg $protein^{-1}$. Results also show that whereas the activity of the Carlsberg subtilisin declined 38.5 and 70.9% with respect to the initial value when preincubated in the cold for 24 or 48 h, respectively, AsES

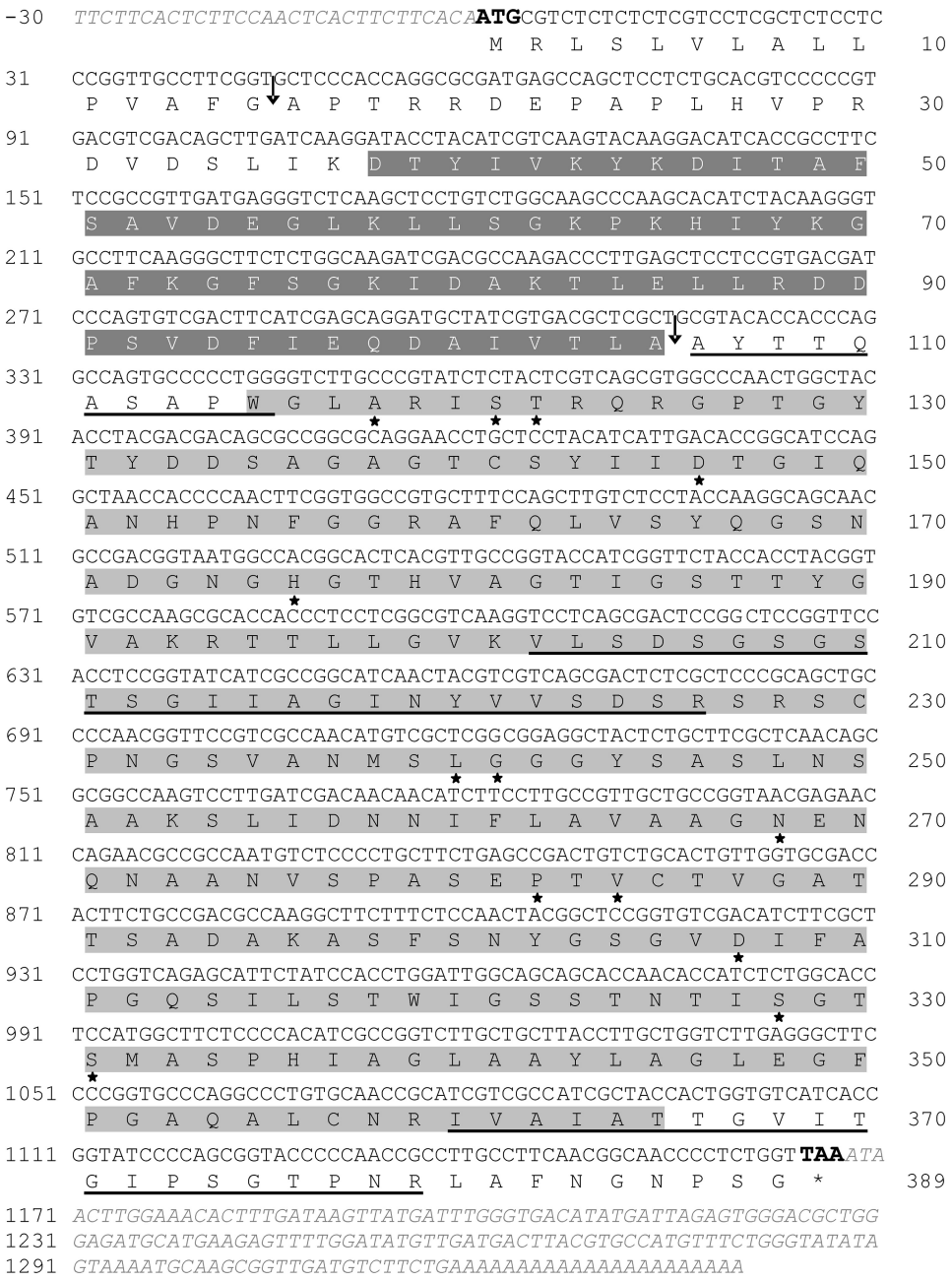

FIGURE 4. **Elicitor-coding full-length cDNA sequence, deduced protein sequence, and predicted structural domains.** Nucleotides and amino acid residues are numbered from the first ATG codon of the transcript and the first methionine of the protein, respectively. The termination codon TAA is marked with *asterisk*. The putative site of signal peptide cleavage and the start of the mature protein are indicated by *arrows*, separating the signal peptide (15 amino acids, Met[1]–Gly[15]) predicted with the SignalP program, a pro-peptide (90 amino acids, Ala[16]–Ala[105]), and the mature protein (283 amino acids, Ala[106]–Gly[388]). The 373 amino acids of the pro-protein and the 283 amino acids of the mature protein are indicated. The *dark shaded letters* indicate the peptidase inhibitor I9 domain (Asp[38]–Ala[105]; E value, 2.44 e-11) and *light-gray shaded letters* cover the peptidase S8 domain or subtilase clan (Trp[115]–Thr[365]; E value, 8.43 e-76), both identified by CD-search. *De novo* determined peptide sequences are *underlined* in the mature deduced protein. Catalytic triad (Asp[41], His[71], and Ser[226]), amino acids of the active site (Leu[135], Gly[136], Asn[163], and Ser[223]), and amino acids of calcium-binding putative sites I (Pro[177], Val[179], and Asp[202]) and II (Ala[13], Ser[16], and Thr[17]) are indicated with *. Untranslated regions (5′ and 3′) are indicated with *gray letters in italics*.

exhibited 15.4% reduction of its proteolytic activity during the first 24 h, but then remained constant until 48 h (Fig. 5). These outcomes indicate that the autoproteolysis exhibited by AsES is much lower that the Carlsberg subtilisin, and therefore the elicitor subtilisin is more stable than the latter. Also, when the elicitor protein was incubated with the substrate N^{α}-benzoyl-DL-Arg-*p*-nitroanilide hydrochloride, which can be cleaved only by chymotrypsin, it was not hydrolyzed (data not shown), demonstrating that AsES possess subtilisin-like proteolytic activity on Suc-AAPF-*p*NA.

The effects of serine, cysteine, aspartyl, and metalloproteases inhibitors on the proteolytic activity of AsES and control Carlsberg subtilisin are summarized in Table 1. With regard to serine proteases inhibitors, the Suc-AAPF-*p*Na hydrolyzing activity of elicitor and control proteases were both completely inhibited by PMSF as expected for all subtilisins. The elicitor proteolytic

activity was also significantly reduced by aprotinin (37% residual activity) and to a lesser degree by leupeptin (54% residual activity) and ovomucoid trypsin inhibitor (65% residual activity), similar to that observed with control subtilisin, whose activity was decreased to 40–68% by action of this three inhibitors.

Therefore, *p*-aminobenzamidine moderately suppressed the protease activity of Carlsberg subtilisin (62% residual activity), whereas on the contrary brought about a strong inhibition on the elicitor protease, reaching a 26% residual activity. On the other hand, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone, a specific inhibitor of chymotrypsin-like serine proteases, did not affect any of the tested proteases as expected, confirming that AsES is a subtilisin. The inhibition degrees exerted by cysteine, aspartyl, and metalloproteases inhibitors on elicitor and control enzymes are almost identical. Slight effects were observed on AsES when cysteine and aspartyl proteases inhibitors were used, yielding identical inhibition percentages (*e.g.* iodoacetic acid and pepstatin A) or even lower (*e.g. N*-ethylmaleimide) than the control protease (Table 1). Metalloproteases inhibitors exhibited a variable effect, being 1,10-phenanthroline more effective than EDTA to decrease the proteolytic activity of both enzymes, although AsES showed lower sensitivity than the subtilisin used as reference (Table 1).

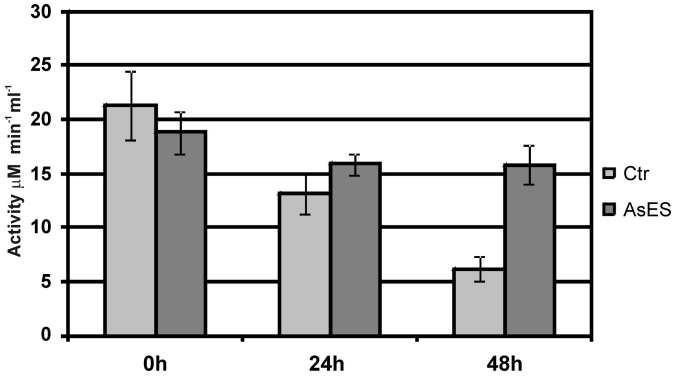

FIGURE 5. **Proteolytic activity of AsES and the Carlsberg subtilisin at different times.** The activity was evaluated with the chromogenic peptide Suc-AAPF-*p*NA. Each protease sample was incubated with the substrate at 37 °C and pH 7.5 for 30 min after a preincubation of the protease solution in the cold (4 °C) for 0, 24, and 48 h. The chromogenic reaction product (*p*NA) was measured at 405 nm. Specific activities of elicitor protease at 0, 24, and 48 h correspond to 128, 108, and 107 μmol min^{-1} mg protein^{-1}, respectively, whereas those of control protease at the same times correspond to 53, 33, and 15 mmol min^{-1} mg protein^{-1}, respectively. Each value is the mean (±S.D.) of three independent replicates.

Because AsES also exhibited proteolytic activity *in vitro*, we incorporated this measurement using the Suc-AAPF-*p*NA as substrate, at each purification step of the elicitor subtilisin. Thereby, the previously described purification protocol of AsES let us to reach a yield of 113% with about 27-fold purification from 4 liters of *A. strictum* SS71 culture (data not shown). The latter represents a recovery of 0.21 mg of pure elicitor protein per liter of culture.

Effect of PMSF on the Resistance Eliciting Activity of AsES—With the aim to test whether the protection effect exerted by AsES against anthracnose on strawberry plants requires its proteolytic activity, PMSF-treated AsES (AsES + PMSF) was used to spray plants before being inoculated with the M11 isolate. As shown in Fig. 6, plants pretreated with AsES + PMSF reached DSR = 5, similar to those pretreated with diluted PMSF solution used as negative control, indicating that the eliciting activity was completely eliminated by inhibiting the proteolytic activity. This biological result further demonstrates that the AsES subtilisin is the one that possess elicitor activity and indirectly confirms the absolute purity of the protein preparation (pool 7 of PS-FPLC). It worthwhile to mention that PMSF is an irreversible inhibitor of serine proteases, so that AsES remained inhibited after removal of PMSF from the reaction medium.

To test whether others subtilisin-like proteinases (such as *T. album* proteinase K and Carlsberg subtilisin) can also induce protection against anthracnose, strawberry plants were pretreated with such enzymes under the same conditions as AsES (2.5 μg ml^{-1}), and results indicate that none of the subtilisins tested, except AsES, has resistance eliciting activity (DSR = 5; Fig. 6).

TABLE 1

Effects of protease inhibitors on the proteolytic activities of AsES and the Carlsberg subtilisin

Enzyme samples were preincubated with each inhibitor at the indicated final concentration at 37 °C for 1 min, and quantitative assay of the proteolytic activity against Suc-AAPF-*p*NA was carried out as described under "Experimental Procedures." The abbreviations used are as follows: Aprot, aprotinin; TPCK, *N*-*p*-tosyl-L- phenylalanine chloromethyl ketone; Leup, leupeptin; NH_2Benz, *p*-aminobenzamidine; OvTI, ovomucoid trypsin inhibitor; NEM, *N*-ethylmaleimide; IAA, iodoacetic acid; Pepst, pepstatin A; 1,10-Phen, 1,10-phenanthroline.

Inhibitor	Specificity	Final concentration	Residual activity[a]	
			Control	AsES
			%	
None			100[b]	100[b]
PMSF[c]	All serine proteases and papain	1 mM	0.1 + 1.1	0.0 + 1.0
Aprot	All serine proteases except thrombin or factor Xa	0.1 mg ml^{-1}	40.1 ± 7.2	36.9 ± 1.0
NH_2Benz	Serin proteases such as trypsin, thrombin, and plasmin	1 mM	62.3 ± 1.3	25.6 ± 5.1
OvTI	Trypsin and partially chymotrypsin	0.1 μg ml^{-1}	68.6 ± 0.9	63.2 ± 5.1
TPCK	Chymotrypsin-like serine proteases (not trypsin)	0.1 mM	100.0 − 2.0	95.6 ± 2.0
Leup	Serine proteases (trypsin, chymase, and plasmin) and cysteine proteases (calpain, cathepsin B, H, and L and papain)	0.1 mM	40.7 ± 6.2	53.7 ± 1.5
NEM	Cysteine proteases	1 mM	76.1 ± 6.0	90.9 ± 1.0
IAA	Cysteine proteases	1 mM	79.5 ± 3.9	82.4 ± 6.9
Pepst	Only aspartic proteases such as renin, chymosin, pepsin, cathepsin D, and protease B	1 μM	83.3 ± 3.0	87.7 ± 3.0
EDTA[c]	Metalloproteases	10 mM	71.2 ± 3.1	92.0 ± 2.0
1,10-Phen[c]	Metalloproteases	10 mM	21.2 ± 3.4	44.1 ± 1.6

[a] Residual activity is expressed as the percentage of the 100% activity under the same assay conditions. Values are the means (±S.D.) of three replications.
[b] 100% of activity corresponds to 13.1 μmol min^{-1} ml^{-1} for the Carlsberg subtilisin and 15.9 μmol min^{-1} ml^{-1} for the AsES and was evaluated after 30 min of incubation at 37 °C in absence of inhibitors.
[c] Broad spectrum inhibitor is indicated.

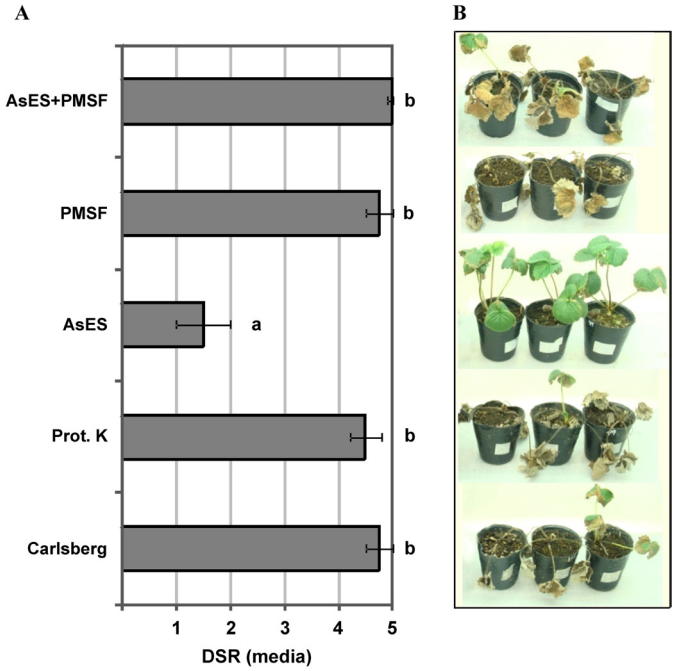

FIGURE 6. **Influence of the proteolytic activity on the protection against anthracnose in strawberry plants.** *A,* resistance eliciting activity of AsES inhibited by PMSF (*AsES+PMSF*) and other subtilisins (fungal proteinase K and bacterial Carlsberg). DSR were evaluated at 21 dpi on cv. *Pájaro* plants sprayed on one leaf with each of the tested enzymatic solutions (2.5 μg ml^{-1}) and 7 days later infected with the M11 isolate of *C. acutatum*. Control corresponded to plants pretreated with PMSF. *Different letters* represent statistically different DSR values (Tukey test, $p = 0.05$). *B,* aspect of cv. *Pájaro* plants pretreated with AsES, AsES+PMSF, proteinase K, Carlsberg subtilisin, or PMSF at 21 dpi with the M11 isolate. Photographs show three representative plants of each treatment.

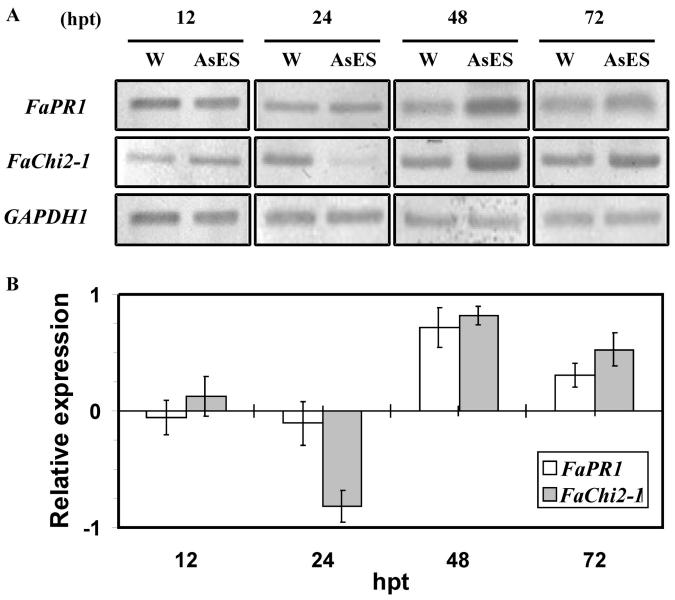

FIGURE 7. **Expression of *FaPR1* and *FaChi2-1* genes in strawberry plants of cv. *Pájaro* treated with AsES at different time point after treatment.** *A,* semi-quantitative RT-PCR assay on leaves of plants sprayed with elicitor protein solution (AsES) or water (*W*) sampled at indicated hpt. *GAPDH1* gene was used as loading control. *B,* relative expression of *FaPR1* and *FaChi2-1* genes after AsES treatment, calculated as the ratio of gene expression change in treated with respect to control plants. Results correspond to average values coming from four replicates and two independent experiments ($n = 6$) with ±S.D.

Elicitation of Strawberry Defense Genes by AsES—To investigate whether the protection effect exerted by AsES is accompanied by the expression of genes associated with defense response activation, *FaPR1* and *FaChi2-1* genes were analyzed in strawberry plants treated with AsES. As shown in Fig. 7, *FaPR1* expression was clearly induced at 48 hpt with AsES compared with control plants, whereas expression of a class II chitinase (*FaChi2-1*) showed a down-regulation at 24 hpt, followed by an evident up-regulation at 48 and 72 hpt compared with control.

Protection Effect against Gray Mold Induced by AsES in Strawberry Plants—Plants pretreated with AsES exhibited higher resistance to B1 isolate of *B. cinerea* (*AsES+B1*) as compared with water-pretreated control plants (*W+B1*) that showed severe symptoms of necrosis (Fig. 8). Plants that were only sprayed with AsES evidenced no symptoms (Fig. 8).

Induction of Defense Responses in A. thaliana by AsES—Results reveal that AsES-treated plants evidenced a strong and transient accumulation of O_2^- and H_2O_2 at early times exhibiting a maximum production of both ROS at 4 hpt (Fig. 9, *A* and *B*, respectively), whereas on the contrary, ROS are not produced at detectable levels in control plants sprayed with water (Fig. 9, *A* and *B*). Also, callose was clearly visualized from 3 dpt in plants sprayed with AsES reaching a maximum accumulation at 9 dpt (Fig. 9*C*), which was 22-fold higher than achieved by the control plants (Fig. 9*D*).

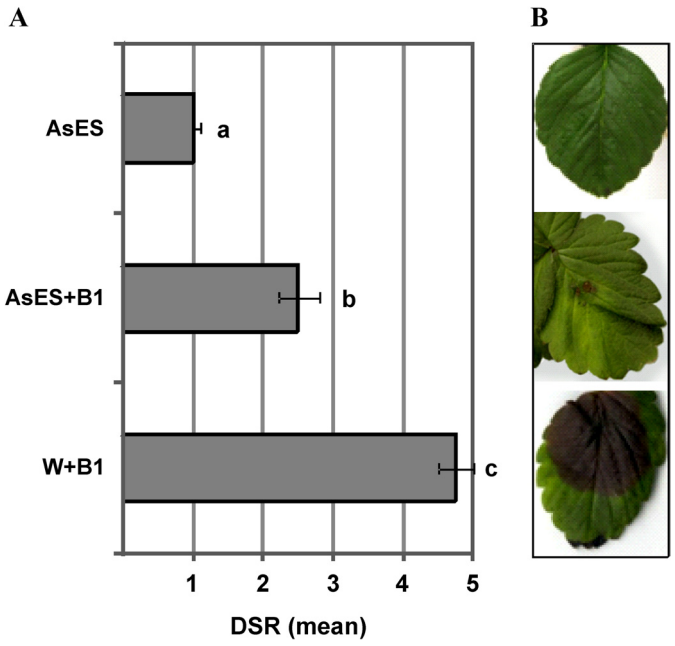

FIGURE 8. **Protection effect of AsES against gray mold in strawberry plants.** *A,* DSR were evaluated at 10 dpi on cv. *Pájaro* plants sprayed with AsES (2.5 μg ml^{-1}) and 7 days later inoculated with the isolate B1 of *B. cinerea* (*AsES+B1*). Controls corresponded to plants sprayed only with AsES or water and then inoculated with B1 (*W+B1*). *Different letters* represent statistically different DSR values (Tukey test, $p = 0.05$). *B,* aspect of cv. *Pájaro* leaves treated with AsES, AsES+B1, and W+B1. Photographs were taken at 10 dpi with B1 isolate and correspond to one leaflet of each experiment. Experiments were carried out with five plants per treatment and were repeated three times.

DISCUSSION

In this paper, we report a novel elicitor protein named AsES that was obtained and purified from *A. strictum* cultures. It was

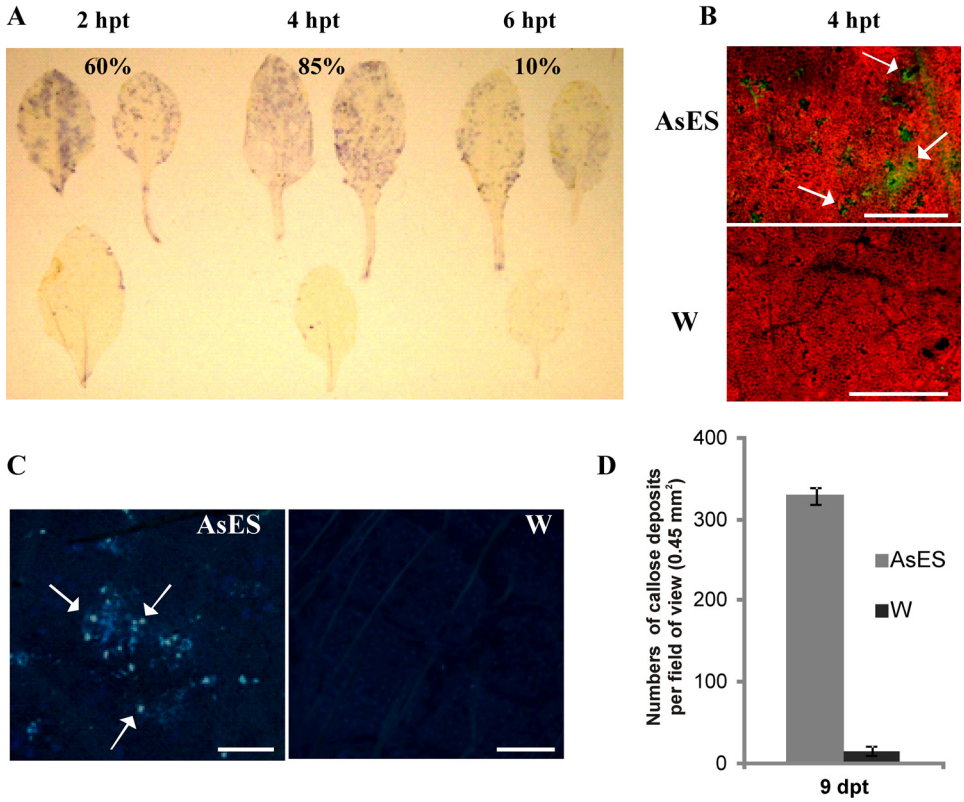

FIGURE 9. **Defense responses induced by AsES in *Arabidopsis* leaves.** Oxidative burst and callose deposition were analyzed in foliar tissues of plants sprayed with AsES or water (*W*, negative control). *A,* accumulation of superoxide radical ($O_2^{\cdot-}$) was visualized as blue precipitates by NBT staining. Photograph shows an example of the 20 leaflets analyzed coming from five plants. *B,* hydrogen peroxide (H_2O_2) and *C,* callose accumulation were stained with 2′,7′-dichlorofluorescein diacetate or aniline blue, respectively. H_2O_2 was observed under fluorescence microscopy as *green spots* and callose as *white dots* (indicated by *arrows*). Each micrograph represents an example of the 20 tissue sectors analyzed coming from five plants taken at 4 hpt or 9 dpt for the accumulation of H_2O_2 or callose, respectively. *Scale bars* in *B* correspond to 10 μm and in *C* to 20 μm. *D,* average numbers of callose deposits per field of view (0.45 mm^2). *Error bar* represents $\pm$S.D. values from four leaves of each plant and five independent experiments. Differences between the response to AsES and control plants were significant as assessed by Student's *t* test at $p < 0.001$.

characterized as a subtilisin-like serine protease that belongs to the proteinase K-like family (50), and it shows extensive similarity to the widely known *T. album* proteinase K (51). AsES is synthesized as a 388-residue preprotein precursor, which undergoes proteolytic removal in two steps. A first event consists of the cleavage of the signal peptide that tags the protein to the extracellular medium, and the second event consists of the removal of the propeptide (inhibitor peptidase I9 domain). Subtilisin propeptides are known to function as intramolecular chaperones, assisting in the folding of the mature peptidase (52), but they have also been shown to act as "temporary inhibitors" of the enzyme function because they are gradually removed by autoproteolytic cleavage activating the enzyme (53). The mature and active protein therefore consists of a 283-amino acid polypeptide of 34 kDa comprising only the catalytic peptidase S8 domain, which in addition to the plant defense eliciting activity retains the protease activity. Because the purified AsES lacks glycoside residues, other post-translational modifications occurring in the protein may cause its aberrant mobility on SDS-PAGE and would explain the discrepancy between the experimental molecular mass and that predicted (28.187 kDa). A recombinant subtilisin from *Pyrococcus furiosus* has also been described to display aberrant mobility on SDS-gel due to high Glu/Asp content (54).

AsES has four Cys residues that probably form two disulfide bonds, which would contribute to the protein stability similarly to proteinase K (51). Although two putative Ca^{2+}-binding sites have been detected in the mature elicitor protein, the lack of inhibition exerted by EDTA and the inhibitory effect exerted by 1,10-phenanthroline on its protease activity suggest that AsES may require the association with Fe^{2+}.

Additionally, among the proteins homologous to AsES include the nonpathogenic subtilisins secreted by endophytic and saprophytic fungi and the pathogenic subtilisins produced by fungal pathogens of plants, nematodes, and insects, whose amino acid sequence identity varies between 55–65% (data not shown). Particularly, subtilisin-like proteinases are considered to be important virulence factors in the infection process of entomopathogenic, nematophagous, and mycoparasitic fungi (55, 56), but little is known however about those produced by plant pathogenic fungi. *Magnaporthe poae*, a fungal pathogen of Kentucky bluegrass, expressed a subtilisin-like proteinase (Mp1) in infected roots, and its expression level correlated with the higher severity of disease symptoms (57). Also, Olivieri *et al.* (58) reported an extracellular subtilisin-like serine protease produced by the phytopathogenic fungus *Fusarium solani* f. sp. *eumartii* that is able to degrade potato PR proteins as well as

specific polypeptides of intercellular washing fluids and cell wall proteins from potato tubers.

AsES also shows similarity (although at lower degree) with the other serine proteases identified in *Acremonium* spp. (48, 49). A serine alkaline endoproteinase with a bound carbohydrate (At1) was purified from leaf sheath tissue of grass *Poa* species infected with the pathogen *Acremonium typhinum*, suggesting that its expression may be involved in the symbiotic interaction between the plant and the fungus (59). Liu *et al.* (48) reported the only two subtilisin-like serine proteases from *Acremonium* spp. that were biochemically characterized, namely AS-E1 of 34.4 kDa and AS-E2 of 32 kDa, but only their N-terminal regions were sequenced. These authors have also reported that both enzymes were able to proteolytically activate prothrombin to meizothrombin(desF1)-like molecules and inhibit plasma clotting, possibly due to the extensive degradation of fibrinogen (48). An important conclusion we can arrive at is that although these proteases are homologous to AsES, none of them exhibits biological activity in plants or are involved in mechanisms of virulence or defense in other organisms.

Because preliminary experiments showed that the active fraction was susceptible to heat and protease digestion (data not shown) suggesting that the elicitor molecule was a protein, two-dimensional gels yielded a single protein, and the MS/MS analysis did not display any strange peptide sequence or other spurious MS peak, we concluded that the AsES protein was pure. Sensitivity of elicitor activity to PMSF demonstrated that the AsES subtilisin is the one that exhibits such activity; however, the expression of AsES in a heterologous system would provide a direct link between the cloned cDNA and elicitor activity of its cDNA product.

Because the first barrier that invading fungi have to overcome is the plant cell wall, fungal endohydrolytic enzymes have been suggested to act as elicitors of the PAMP-type (10). The latter could also occur with the AsES as it is able to induce a defense response in different plant species. Our results clearly show that AsES can trigger a strong defense reaction in strawberry, which is characterized primarily by a transient oxidative burst, then by a strong transcriptional induction of *PR-1* (*FaPR1*) and class II chitinase (*FaChi2-1*), and finally manifested by an enhanced resistance against fungal pathogens of hemibiotrophic (*i.e. C. acutatum*) and necrotrophic (*i.e. B. cinerea*) lifestyle. The fact that the protection effect mediated by AsES was not restricted to pathogens of the genus *Colletotrichum*, let us further conclude that AsES is able to induce a broad-based resistance against pathogens (5).

Synthesis of proteins directly associated with defense response in plants, as PR proteins, is essential to protect them against pathogen infection (10). Hence, the induction of PR genes such as *PR-1, PR-2* (including β1,3-glucanases), *PR-3* (including class II chitinases), among others, is clear evidence of defense mechanism activation (60). Furthermore, it is well documented that the increase of the expression of *PR-1* is strongly associated with systemic acquired resistance activation in pathogen-challenged or elicitor-treated plants, whereas induction of glucanase and chitinase genes is closely related to systemic defense activation against a broad pathogen spectrum (60, 61). Particularly, the induction of many PR genes has been reported in strawberry plants challenged by pathogens (62).

Further studies revealed that AsES can also confer protection to different strawberry cultivars against different virulent isolates of *C. acutatum* (data not shown). We may also speculate that the production of ROS (*i.e.* H_2O_2 and O_2^-) and the cell wall reinforcement due to callose deposition observed in *Arabidopsis* plants would also confer resistance to pathogens as suggested elsewhere (7, 10, 12).

Our results suggested that the protease activity of AsES is essential for its elicitor function in strawberry plants. However, elicitor activity of another hydrolytic enzyme as the *Trichoderma viride* endoxylanase, a PAMP that elicits hypersensitive response, ethylene, and phytoalexin production in tobacco and tomato, was found to be independent of enzyme activity (63). Moreover, in few cases elicitor activity was found to be determined by small fragments of the intact elicitor molecule of PAMP, suggesting recognition of "epitope"-like structures by receptors at the plant cell surface (64). Examples of the latter are an 8-amino acid glycopeptide fragment derived from yeast invertase (gp 8c) (65) and an internal peptide of 13 amino acids (Pep-13) derived from 42-kDa cell wall glycoprotein of *P. sojae* that exhibits Ca^{2+}-dependent transglutaminase activity (19, 66).

Defense inducing activity was detected in some proteases derived from both bacterial and fungal pathogens (12). In phytopathogenic bacteria of the genera *Pseudomonas*, *Xanthomonas, Ralstonia,* and *Yersinia*, four families of cysteine proteases (*i.e.* XopD, YopJ, YopT, and AvrRpt2), including many "effector" proteins, have been identified (67). In fungi, however, a single group of proteases with defense eliciting activity was identified. Cultivar-specific *Avr-Pita* genes have been cloned and characterized from *Magnaporthe oryzae* (formerly known as *Magnaporthe grisea*) that is the causal agent of blast rice. *Avr-Pita* gene encodes a secreted 223-amino acid preprotein with homology to fungal zinc-dependent neutral metalloproteases that is further processed into an active 176-amino acid mature protein (68). Point mutations in the putative protease catalytic residues of Avr-$Pita_{176}$ resulted in gain of virulence on rice cultivars carrying the cognate resistance gene *Pi-ta*, although a direct biochemical evidence for protease activity of Avr-Pita is still missing (64, 69). Furthermore, mutations in the putative catalytic residues of AvrBsT, a bacterial avirulence effector that displays structural similarity to the YopJ family of cysteine proteases, also abolished avirulence activity (70). We therefore hypothesized that AsES may function by releasing an active elicitor from a plant precursor molecule rather than being itself an elicitor. The latter has been described for the gene *avrD* from *Pseudomonas syringae* pv. *tomato*, whose product is responsible for the synthesis of syringolide elicitors (71). Furthermore, some peptides that are considered elicitors, such as systemin, HypSys (hydroxyproline-containing glycopeptides), and RALF (rapid alkalinization inducing factor), would apparently come from protein precursors constitutively present in plant cell wall or cytoplasm and would be activated by microbial proteases or by intracellular proteases upon cell injury, acting as elicitors of the DAMP-type (72).

Because the elicitor activity was only found in AsES from SS71 *A. strictum* and not was found in a homologous subtilisin as proteinase K, we conclude that the proteolytic activity is necessary but not sufficient to induce defense, and we suggest that AsES might induce defense by means of proteolysis of one or multiple host proteins that are specific targets of this protease.

Although the results presented in this paper confirm that the proteolytic activity exhibited by the AsES elicitor is required for the induction of the defense response in plants, further experiments using recombinant AsES protein, site-directed mutants, or synthetic (PAMP-like) peptides derived from AsES are necessary to elucidate its action mechanism and the resistance-causing signal perception process.

In conclusion, we have purified and characterized AsES, a novel extracellular elicitor protein produced by the pathogenic fungi *A. strictum*, which exhibits defense inducing activity on its strawberry host *Fragaria* × *ananassa* and other nonhost species *A. thaliana*, and *in vitro* subtilisin-like proteolytic activity. AsES can be considered a new member of the fungal protein effectors. Despite the variety of examples showing that fungal proteases are involved in plant defenses, no subtilisin-like protease from a phytopathogen has been reported so far. This discovery is relevant in the plant-pathogen interaction knowledge and may contribute to envisioning possible strategies for controlling diseases in the field.

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